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FACTORS AFFECTING FORMATION AND FUNCTIONS OF APPRESSORIA OF *COLLETOTRICHUM GRAMINICOLA*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Factors Affecting Formation and Functions of Appressoria of Colletotrichum graminicola", submitted by Hans J. Netolitzky in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Appressoria of Colletotrichum graminicola form at the tips of short germination tubes within 24 hours on the surface of hosts or in artificial culture. Germination of conidia and formation of appressoria occurred in a large variety of nutrients, including: carbohydrates, sulfur-containing and related compounds, inorganic nitrogenous compounds, amino acids, peptides and proteins. The only compounds that strongly inhibited appressorial formation were those which contained sulphydryl group(s) or those, which had the potential to form these groups. Thus, the following nutrients were highly effective as inhibitors of appressorial formation: cysteine, homocysteine, thiohistidine, tripeptide glutathione, 2-mercaptoethylamine, peptones, some proteins, penicillin (a sulfur-contining antibiotic) and selenium and tellurium (members of the sulfur family). However, the oxidized forms of cysteine (cysteine sulfinic acid, cysteic acid) and oxidized glutathione were not inhibitory. The inhibitory effect on appressorial formation, was lost when the hydrogen on the sulfur of cysteine was replaced by a methyl group, as in S-methyl cysteine. Three possible mechanisms of action of these compounds in suppressing appressorial formation are discussed.

There was no evidence that hydrogen-ion concentration or carbon:nitrogen ratios appreciably affected appressorial formation.

The contact stimulus was a factor influencing appressorial formation. However, appressoria were formed only on harder surfaces,

for example, on glass slides, glass wool or on harder gelatin membranes. Soy peptone inhibited appressorial formation even though the contact stimulus was present.

Details of the process of penetration of barley leaves are described, and were found to be similar to the penetration process occurring in other species of the genus Colletotrichum.

The age of the leaf of barley and temperature were the two factors influencing penetration. The inoculated leaves, incubated at 20° C, were initially susceptible, followed by a period of resistance and then susceptibility again. The period of resistance varied with light intensity, which appears to be associated with the rate of ageing.

The effect of temperature appeared to be dominant over the age-of-leaf factor in influencing the act of penetration. Inoculated barley leaves, incubated at 30° C, were readily penetrated within 72 hours, even during the period of time that these leaves were resistant when incubated at 20° C.

Appressoria of C. graminicola can function as organs of survival as well as organs of penetration. They possess the following characteristics of chlamydospores: thick-walls, ability to withstand long periods of desiccation, and ability to produce germ tubes.

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INTRODUCTION

Appressoria are organs of penetration in a large number of fungi. In the early literature they have been referred to as "holdfasts" because they adhere to the plant surface by means of a mucilaginous substance. They vary in size, shape, color, cell wall thickness and cellular structure, but these characteristics remain fairly constant within any fungal species.

Colletotrichum graminicola (Ces.) Wils, the incitant of anthracnose of graminaceous hosts, penetrates by means of appressoria, which under favourable conditions are produced abundantly in artificial culture or on plant material within a relatively short period of time. The morphological features of their development have been described by Skoropad (48). An appressorium is initiated as a terminal swelling of the germ tube from which it is soon separated by a septum. It is first club-shaped, but soon broadens and develops a thick, brown wall. Mature appressoria are globose, pear-shaped or irregularly lobed. However, distinctive characteristics within this species are retained. Sutton (50) has proposed that isolates of C. graminicola from different hosts could be separated on morphological differences in their appressoria.

Frank (15) described appressoria in 1883 and demonstrated that these organs acted as holdfasts for the early stages of infection. He was the first to recognize the nature of these structures and called them appressoria or adhesion organs. Halstead (19), in 1893, showed that 24 fungal-species, belonging to the "anthracnoses", produced appressoria. He found that a rich medium favoured mycelial growth but few appressoria were formed under these conditions. Halstead's work was confirmed by

Hasselbring (21) who, in 1906, conducted a detailed study on appressorial formation.

De Bary (8) was the first to propose that the stimulus for the production of appressoria was mechanical contact. Halstead (19) and Dey (10) confirmed the observation that appressoria formed only when in contact with a hard surface. In many host-parasite combinations appressoria have been shown to form preferentially at junctions of epidermal cells (9, 21, 42, 34).

More recently, other factors have been shown to affect appressorial formation. Van Burgh (52), using conidia of Colletotrichum phomoides sown on formalized gelatin membranes of graded hardness, found that appressoria were not formed on the softest membrane, but their numbers increased on membranes of increasing hardness. The addition of certain kinds of nutrients prevented the formation of appressoria even on the harder membranes. Hurd-Karrer and Rodenhiser (23) reported the opposite effect of nutrients when they found that appressoria of several Puccinia spp. were formed on agar containing glucose and mineral nutrients but were not observed on water agar. Sharp and Smith (46) found that in P. coronata appressorial formation was as high as 40 per cent when gelatin was used as a substrate and zinc was supplied as $ZnSO_4$, but on gelatin without zinc very few appressoria were formed. Dickinson (11) studied P. triticina on paraffin and collodion membranes. He found that numerous appressoria were formed on membranes made from hard wax but none was formed on membranes made from soft wax. They were formed on collodion membranes when cell fragments were incorporated with the collodion. Conidia, germinating on portions of the leaf with the mesophyll exposed by the removal of the epidermis, did not form appressoria.

Kerr and Flentje (29) showed that the presence of a cuticle was important in appressorial formation in Pellicularia filamentosa. Infection cushions were not formed on exposed cortical cells.

Allen (1) isolated from uredospores of P. graminis var. tritici a volatile fraction that stimulated germination, inhibited germ tube differentiation, and brought about differentiation resulting in appressorial formation. Recently Dunkle, Maheshwari, and Allen (12) reported that inhibitors of RNA synthesis (actinomycin D and 5-fluorouracil) interfered with appressorial formation, but did not interfere with germination and growth of the germ tube. Inhibitors of protein synthesis (puromycin and p-fluorophenylalanine) did not prevent appressorial formation but prevented further differentiation into vesicles and infection hyphae. These authors indicated that the initiation of infection structures requires the synthesis of RNA and complete development of infection structures requires protein synthesis.

There appear to be a number of factors required for the formation of penetration organs in some fungi. Flentje (13), working with Pellicularia filamentosa, found two distinct reactions related to the formation of penetration organs. The first is the reaction governing growth of the main hyphae and the other is the inhibition of growth and formation of side branches on which infection cushions form. Strains of this fungus growing on susceptible plants to which this particular strain was not pathogenic, did not form any penetration organs. Instead of producing side branches and infection cushions, the mycelium grew irregularly over the surface without adhering to it, or the mycelium adhered to the surface and followed the lines of juncture of the epidermal cells. A hypothesis proposing contact only for the stimulus for the production of penetration organs was not adequate and chemical stimulus was suggested.

Kerr (28) and Kerr and Flentje (29) also investigated this phenomenon using seedlings grown in cellophane bags and subsequently buried in soil inoculated with various strains of P. filamentosa. Hyphae of pathogenic strains of this fungus aggregated opposite roots of susceptible hosts, but did not do so in the case of resistant hosts. This indicated that there was a diffusible substance acting through the cellophane. The diffusible material was collected and the response of the fungus was studied. Extract from a susceptible host caused hyphal elongation to cease and side branches to proliferate, giving a clumping similar to infection cushions. In controls without the root exudate, the mycelium grew at random across the cellophane. On epidermal strips from stems the hyphae became attached to the surface and grew along the lines of junction of cells but no infection cushions were formed. Infection cushions were formed when the exudates from the susceptible host were included.

Brown and Harvey (5) used Botrytis cinerea on gold foil, on epidermis of Allium scales, and on paraffin and gelatin membranes, and concluded that the stimulus for appressorial formation was contact. They showed that the chemical composition of the medium under paraffin was of no significance to penetration.

Purdy (43) reported that an external carbon source was essential for the formation of appressoria in Sclerotinia sclerotiorum. Mechanical contact was also necessary, as appressoria formed only when in contact with the cover slip.

Maheshwari et al (31) showed that contact with the isolated cuticle was the only requirement to promote appressorial formation in a number of Puccinia species. Artificial membranes, containing certain hydrocarbons, were also effective in promoting appressorial

formation. The chain length of these hydrocarbons was estimated to be in the order of 30-CH_2 units in the molecule.

Forlot (14) used tomato varieties which were susceptible and resistant to Colletotrichum coccodes and found a strong correlation between resistance and reduction in appressorial formation. Two extracts were obtained: 1) by washing the leaf cuticle with chloroform, and 2) by crushing leaves and passing the extract through a Millipore filter. Conidial suspensions were prepared in these extracts and incubated on glass slides. Very few appressoria formed in the solutions containing the chloroform extracts from resistant plants. The semi-susceptible varieties were intermediate, and a high proportion of spores germinating in extracts from leaves of susceptible varieties formed appressoria. Few appressoria formed in the preparations obtained from crushed leaves of both resistant and susceptible varieties.

Leech (30) described the process of penetration of bean by C. lindemuthianum, and it has generally been accepted as representative of the penetration process in this genus. However, the steps in the penetration process have not been reported in detail for C. graminicola.

Skoropad (48) showed that, in C. graminicola, the act of penetration was influenced by temperatures which were different than those which influenced appressorial formation. He found that appressoria were produced over a wide range of temperatures, 15-35° C, but that penetration was usually restricted to the narrower range of 25-30° C.

It has been suggested that appressoria could function as organs of survival, analogous to chlamydospores. Birachi (3) and Simmonds (47), working with Gloeosporium spp., found that appressoria were resistant to drying and suggested that they might function as chlamydospores. However, Dey (10) found that appressoria of Colletotrichum gloeosporioides were not resistant to drying. Simmonds (47), McOnie (32) and Green (18) reported that appressoria were more resistant than conidia to fungicides. Stanghellini and Aragaki (49), working with C. gloeosporioides on papaya, found that appressoria remained latent on the fruit for some time and were considered an important factor in infection as the fruit ripened.

Host penetration by C. graminicola occurs only by means of appressoria and, therefore, any factors which can inhibit their formation would preclude development of the anthracnose disease. The main purpose of this study was to determine the effect of some nutrients, of certain physical factors, and of hydrogen-ion concentration on the formation of appressoria in this fungus.

Conditions which favor the formation of appressoria are not necessarily conducive to penetration. Therefore, the second purpose of this study was to determine the effect of different light intensities, age of leaf, and temperature on host penetration by C. graminicola.

Since appressoria of this fungus showed certain morphological characteristics of chlamydospores, the third purpose of the investigation was to determine whether they could function as organs of survival.

MATERIALS AND METHODS

A. Factors Affecting Formation of Appressoria

I. Nutrition

(i) Fungus

The isolate of C. graminicola, used in these studies, was obtained from wheat stubble in the Edmonton area. Stock cultures originating from a single conidium were maintained on potato sucrose agar (PSA) in a large number of test tubes stored at 4° C. This procedure obviated the need of sub-culturing too often during the course of the investigation. Conidia were produced abundantly within 4 days at 20° C on PSA which was seeded with a mixture of conidia and some mycelium suspended in distilled water.

(ii) Production of Appressoria

In order to study the effect of various chemicals on the formation of appressoria, a method of producing them on micro slides was developed. A 90 mm Petri plate was lined with moistened filter paper. A V-shaped piece of glass tubing, placed on the filter paper, supported a standard glass micro slide. This arrangement provided a moist chamber in which evaporation of water placed on the slide was reduced to a minimum during the period of incubation.

Distilled water was used in the preparation of all chemical solutions. Since conidia were added to these solutions in a suspension of distilled water, this additional water was taken into account in the calculation of the concentration of chemicals in each solution. A 0.3 ml aliquot of the solution, containing conidia in suspension, was pipetted onto each slide and spread across it. In this

preparation a density of 3-4 conidia in a low power field (x100) of a microscope was considered desirable. Higher densities of conidia made it difficult to locate the source of individual hyphae.

The cultures were incubated at 20° C for 24 hours in closed Petri plates. Under favorable conditions appressoria were produced abundantly within this period of time. Following incubation the cultures were air-dried and stained with cotton blue in lactophenol or in acid fuchsin in lactic acid.

A medium consisting of 1 percent sucrose in distilled water was highly stimulatory to the germination of conidia and to the production of appressoria. The basis on which this medium was selected for these qualities is evident from the results presented under the section dealing with the effect of carbohydrates on the formation of appressoria (p. 23).

The 1 percent sucrose solution was used: (1) to check the ability of each new batch of conidia to produce appressoria, and (2) as a basal medium in which chemicals lacking a source of carbohydrate were prepared.

Chemicals which were tested for their effect on the formation of appressoria in C. graminicola were selected from the following groups: carbohydrates, nitrogenous compounds, fatty acids, and sulfur-containing and related compounds.

In another series of experiments different concentrations of sucrose were used in combination with different concentrations of nitrogenous compounds to determine if the C:N ratio might have an effect on appressorial formation.

Another combination of nutrients consisted of L-cysteine HCl and one of the several nitrogenous compounds.

Each experiment consisted of 8 micro slides repeated at 2 different times. Conidia were rated as either having produced appressoria or mycelium only. Germination was considered to have occurred when the germ tube was at least as long as the conidium. One hundred germinating conidia were observed on each slide, and the final rating was recorded as the average percentage of conidia producing appressoria.

The chemicals used and minor modifications in methods are listed under each group.

(iii) Carbohydrates

Each of the following carbohydrates was used at a concentration of 1 percent in distilled water: D glucose, D(+) mannose, D(-)xylose, D galactose, D galacturonic acid, D fructose, D mannitol, sucrose, D(+)melibiose, D(+)raffinose hydrate, D(+) cellobiose, lactose, soluble starch, dextrin, inulin, xylan, pectic acid, sodium polypectate, polygalacturonic acid, amygdalin and salicin. Sucrose was tested at additional concentrations of 0.1, 0.5, 5.0, 10.0, 20.0, 30.0 and 40.0 percent.

Benzaldehyde and mandelonitrile, the breakdown products of amygdalin, were tested for their effect on formation of appressoria at concentrations of 0.001, 0.01, 0.1 and 1.0 percent in a 1 percent sucrose solution. The presence of HCN during the breakdown of amygdalin was determined by the method using sodium picrate reagent in Obrink's modified Conway microdiffusion dishes with slight modifications in the method described by Colotelo and Ward (6). The procedure

was modified in that 3 ml of sodium picrate reagent were added to the center well and 6 ml of a solution of 1 percent amygdalin and conidia (in the same proportion as in carbohydrate experiments) were added to the outer well. The control was similar but no conidia were suspended in the amygdalin solution. The pH of all solutions was adjusted with KOH to be within the range of 6-7.

(iv) Nitrogenous Compounds

Unless otherwise indicated, all nitrogenous compounds were used at a concentration of 1 percent in a 1 percent sucrose solution.

The following compounds were tested for their effect on the formation of appressoria:

amino acids - glycine, L alanine, L serine, L valine, L leucine, aspartic acid, L citrulline, L asparagine, L cystine, L cysteine HCl, L tyrosine, DL methionine, DL phenylalanine, L proline, and L histidine HCl. (L tyrosine and L cystine were not soluble at a concentration of 1 percent and were used as 0.1 percent solution for tyrosine and approximately 0.01 percent for cystine);

inorganic nitrogen - ammonium phosphate, ammonium sulfate, ammonium nitrate, potassium nitrate, and potassium nitrite;

peptides - glycyl L tyrosine, DL alanyl DL valine, glycyl DL aspartic acid, DL alanyl DL alanine, L phenylalanine DL alanine, glycyl DL serine, DL alanyl DL leucine, glycyl L proline, and glycyl D asparagine;

peptones - Soy Peptone Bacteriological (Fisher Scientific), Bacto-Peptone (Difco), Bacto-Soytone (Difco), Soy Hydrolysate

(Nutritional Biochem. Co.), Peptone-S (Nutritional Biochem. Co.), and Proteose-Peptone (Difco).

Soy Peptone Bacteriological was tested at concentrations of 0.001, 0.01, 0.1 and 1.0 percent. One series was used with 1 percent sucrose and one without sucrose.

proteins - enzymatic proteins: trypsin, crude protease, peroxidase, pronase, alpha amylase, lipase, cellulase, catalase, polyphenol oxidase, glucose oxidase, cytochrome C oxidoreductase, and pectinase (all were obtained from the Nutritional Biochemical Co.).

Proteins were prepared in a 1 percent solution of sucrose. Enzymatic proteins, except trypsin and peroxidase, were used at a concentration of 0.1 percent. Trypsin was prepared at concentrations of 0.005, 0.01, and 0.1 percent, and peroxidase at concentrations of 0.1 and 0.2 percent.

An inactive form of trypsin was obtained by autoclaving a preparation of 0.1 percent trypsin in a 1 percent sucrose solution for 20 minutes at 7.7 kilos (17 lbs.) pressure.

(v) Fatty Acids

The following fatty acids, which are known to be components of plant cuticles, were used: stearic, palmitic, oleic and linoleic. Each fatty acid was prepared in a 1 percent sucrose solution.

The fatty acids, which are highly insoluble in water, were used as saturated solutions.

(vi) Sulfur-containing and Related Compounds

The sulfur-containing and related compounds were prepared in a 1 percent sucrose solution.

Each of the following sulfur-containing compounds was used in concentrations of 0.1, 0.5 and 1.0 percent: potassium sulfate, potassium bisulfite, L cysteine HCl, L cysteine sulfinic acid, L cysteic acid, DL homocysteine, DL homocysteic acid, L thiohistidine, S-methyl cysteine, reduced glutathione, oxidized glutathione, DL methionine sulfone, DL methionine DL sulfoxide, 2- mercaptoethylamine, potassium tellurite, potassium selenite and Penicillin G potassium salt.

Since potassium selenite and potassium tellurite inhibited conidial germination at 0.1 percent, they were used at concentrations of 0.02, 0.002 and 0.0002 M.

(vii) Carbon:nitrogen Ratio

In all experiments concerning the effect of different ratios of C:N on formation of appressoria, the carbon source, sucrose, was used in concentrations of 0.1, 0.5, 1.0 and 5.0 percent. Organic sources of nitrogen, in combination with each concentration of sucrose, were used as 0.1, 0.5 and 1.0 percent solutions, while inorganic sources of nitrogen were used as 0.1, 0.5, 1.0 and 2.0 percent solutions.

The final pH of each solution was adjusted with KOH or HCl to be within the range of 5.5 - 6.5.

Combinations of compounds, which inhibited the germination of conidia at certain concentrations are so recorded in the Tables in Results. Wherever germination of conidia was not affected appreciably, the average percentage or germinating conidia producing appressoria is listed.

The following compounds were used as sources of nitrogen in combination with sucrose:

inorganic nitrogen - KNO_3, KNO_2, NH_4NO_3, and NH_4NO_2;

amino acids - glycine, L alanine, L serine, L cysteine HCl, L threonine, DL methionine, L leucine, L glutamic acid, L arginine, L lysine monohydrochloride, L phenylalanine, L histidine HCl, and L proline;

peptides - DL alanyl DL methionine, glycyl DL serine, glycyl DL leucine, DL alanyl DL phenylalanine, and DL alanyl glycine;

peptones - Proteose-Peptone, Peptone S and Soy Peptone Bacteriological.

(viii) Cysteine Combined with Nitrogenous Compounds

In the combinations of L cysteine HCl and other nitrogenous compounds, used singly, each one was used at a concentration of 0.5 percent in a 1 percent sucrose solution.

Each of the following compounds was used in combination with L cysteine HCl: L proline, DL methionine, glycine, L leucine, L alanine, L histidine HCl, L tryptophane, L serine, L threonine, L-methyl cysteine, L lysine monohydrochloride, L phenylalanine, L glutamic acid, L arginine and ammonium nitrate. L cysteine HCl was also included at 0.5 percent in 1 percent sucrose solution.

The final pH of each medium was adjusted with KOH or HCl to be within 5.5 - 6.5.

II. Hydrogen-ion Concentration

A 1 percent sucrose solution was used as the basal medium. This medium was adjusted to pH's ranging from 1.5 to 12.0 by means of buffer systems (17) listed below:

pH	Acid	Salt
1.5	2.5 ml 0.5 N HCl	0.20 gm KCl
2	0.5 ml 0.5 N HCl	0.28 gm KCl
3	0.67 gm $H_3C_6H_5O_7 \cdot H_2O$	0.08 gm $Na_3C_6H_5O_7 \cdot 2H_2O$
4	0.48 gm $H_3C_6H_5O_7 \cdot H_2O$	0.38 gm $Na_3C_6H_5O_7 \cdot 2H_2O$
5	0.30 gm $H_3C_6H_5O_7 \cdot H_2O$	0.66 gm $Na_3C_6H_5O_7 \cdot 2H_2O$
6	0.14 gm $H_3C_6H_5O_7 \cdot H_2O$	0.94 gm $Na_3C_6H_5O_7 \cdot 2H_2O$
7	0.45 gm $NaH_2PO_4 \cdot H_2O$	1.23 gm $Na_2HPO_4 \cdot 7H_2O$
8	0.06 gm $NaH_2PO_4 \cdot H_2O$	1.91 gm $Na_2HPO_4 \cdot 7H_2O$
9	0.03 gm Na_2CO_3	0.29 gm $NaHCO_3$
10	0.22 gm Na_2CO_3	0.14 gm $NaHCO_3$
11	0.31 gm NH_2CH_2COOH	0.13 gm NaOH
12	0.31 gm NH_2CH_2COOH	0.21 gm NaOH

Each solution was made to a volume of 1 litre with distilled water.

III. Physical Factors

(i) Hanging Drop Preparation

Conidia, suspended in a 1 percent sucrose solution, were mounted in a hanging drop micro slide. The slide was placed on a V-shaped piece of glass tubing in a Petri plate lined with moistened filter paper, and the material was incubated at 20° C. Observations for the presence of appressoria were made at various intervals over a period of three days.

(ii) Membranes of Graded Hardness

Formalized gelatin membranes were prepared according to the method described by Brown and Harvey (5). Five membranes, differing in degree of hardness, were obtained by immersing gelatin sheets for 24 hours in a preparation of 0, 20, 40, 60, or 80 percent ethanol, each solution containing 10 percent formalin. The softest membrane was formed in a solution without ethanol, while membranes of progressively increased hardness were formed in each increased concentration of ethanol. Membranes were soaked and rinsed in several successive changes of sterile, distilled water to remove the formalin. They were placed on glass slides which were set either in Petri plates lined with moistened filter paper, or in Petri plates containing 2 percent water agar.

These membranes were seeded with conidia suspended in distilled water containing 1 percent sucrose or 1 percent Soy Peptone Bacteriological, and incubated at 20° C. Observations for presence of appressoria were made at various intervals following incubation after staining with cotton blue in lactophenol.

(iii) Glass Wool

A mat of glass wool (Fisher Scientific, fine grade) was embedded at, or near, the surface of each of the following preparations: (1) 2 percent water agar,

(2) 2 percent water agar containing 1 percent Soy Peptone.

These media were aseptically seeded with conidia of C. graminicola, suspended in distilled water, and incubated at 20° C.

(iv) Excised Leaves

Moore, CI 7251, a cultivar of barley highly susceptible to C. graminicola, was used.

Leaves were excised and washed in running tap water for 30 minutes. A 2-inch segment from the mid-section of the leaf was inoculated with conidia suspended in distilled water. These leaves were then floated on a solution of 40 ppm benzimidazole and 10 ppm aureomycin, and incubated at 20° C for 72 hours. After the leaves were cleared in acetic acid - alcohol and mounted in cotton blue lactophenol, the location of appressoria was determined by observation with a microscope.

B. Factors Affecting Functions of Appressoria

I. Penetration

Moore, CI 7251, a cultivar of barley highly susceptible to C. graminicola, was used in this study. An isolate of C. graminicola was obtained from wheat stubble, single-spored and maintained in culture on PSA (See section A for details).

Barley plants were grown in a growth chamber at a temperature of 21° C during the 16-hour light period, and 13° C during the 8-hour dark period. Three different light intensities, 1200, 1600 and 2200 foot-candles, were used. They were obtained by adjusting the height of the benches in relation to the light source.

The first and third leaves were harvested, beginning 3 days after they had unfurled. Sampling continued every 3 days until the leaf had aged to a point of turning a uniform yellow. The third

leaf was obtained only from plants grown in a light intensity of 1600 foot-candles.

The method of excising leaves and preparing them for floating on a solution of benzimidazole and aureomycin is described in detail under section A.

The excised leaves were inoculated with conidia of C. graminicola, suspended in distilled water, and held at 20° C for 72 hours in the dark. They were then cleared in an acetic acid - alcohol preparation, as described by Biehn et al (2), and mounted in cotton blue in lactophenol. The extent of penetration, which was determined by observing the leaves with a microscope, was rated on a scale of 0-4, with 0 indicating no penetration and 4, the highest level of penetration. Three leaves of each age were checked at 2 different times.

Since the process of penetration had not been described in detail for this fungus, it was included in this study. Leaves, which were used for histological studies of the process of penetration, were fixed, dehydrated and embedded in tissue mat. They were sectioned at 6-8 μ thickness, and stained with safranin and fast green.

The effect of temperature on penetration of the third leaf during its period of resistance was determined by using barley leaves at 14 days following unfurling. These leaves were prepared for inoculation with conidia of C. graminicola according to the method described above. They were subjected to one of the following conditions before being observed for evidence of penetration:

a. Floated for 48 hours at 20° C, inoculated, and incubated for 72 hours at 20° C.

b. Floated for 48 hours at 30° C, inoculated, and incubated for 72 hours at 20° C.

c. Floated for 48 hours at 30° C, inoculated, and incubated for 72 hours at 30° C.

Three leaves of each age were checked at two different times.

II. Survival

Conidia of C. graminicola, suspended in a 1 percent sucrose solution, were seeded on 18 mm glass cover slips. They were placed on micro slides which were set on V-shaped glass tubing in sterile Petri plates lined with moistened filter paper, and incubated at 20° C for 24 hours. Appressoria, which were produced during this period of time, were air-dried, in situ.

In a second experiment the procedure was similar, with the exception that soy peptone at a concentration of 2 percent was added to the 1 percent sucrose solution in which the conidia were suspended. After incubation the mycelium produced on the cover slips was air-dried.

In the third experiment the conidia were placed on cover slips in a 1 percent sucrose solution and air-dried immediately.

Cover slips from the 3 different treatments were stored in sterile Petri plates at room temperature. The viability of the fungal material was checked by inverting the cover slips on PSA in Petri plates after the appressoria were air-dried for the following periods of time: 1, 3, 6, 9, 21, 28, 35, 42, 49, 100, 200, 300, 400, 450 and 500 days. New growth, as observed under the microscope, was evidence of viability.

Detached appressoria were also checked for their ability to survive various periods of dessication. Conidia were suspended in a 1 percent sterile sucrose solution and seeded in Petri plates. The culture was incubated at 20° C for 72 hours. Following this period of time, conidia which had not germinated or which had not produced appressoria were washed off with a jet of distilled water. Appressoria were then scraped in distilled water from the surface of the glass with a rubber policeman. They were centrifuged at 1800 x G in a Sorval GLC-1 bucket centrifuge for 20 minutes. The pellet of appressoria was washed with sterile, distilled water and resuspended in sterile water. One portion of these appressoria was air-dried immediately on sterile, 18 mm glass cover slips, another portion was seeded on PSA in Petri dishes, and the third portion was used to inoculate excised barley leaves which were floated on a solution of benzimidazole and aureomycin, and incubated at 30° C.

The viability of the air-dried, detached appressoria was determined by placing the seeded cover slips on PSA and checking for new growth.

RESULTS

A. Factors Affecting Formation of Appressoria

I. Nutrition

(i) Basal Medium

A high percentage of germinating conidia of C. graminicola produced appressoria in sucrose solutions at concentrations of 0 to 30 percent (Table 1). In distilled water, and in sucrose solutions of 0.1 to 0.5 percent, a high percentage of germinating conidia formed appressoria but the level of germination was low. There was a progressive decrease in the numbers of appressoria even though the percentage of conidia that germinated was high in sucrose solutions with concentrations above 1 percent and up to 30 percent. Germination was almost completely suppressed in the 40 percent sucrose solution.

Typical appressoria formed in 1% sucrose solution are shown in Fig. 1 and 2. The 1 percent sucrose solution was selected as the basal medium because it was highly stimulatory to conidial germination and to synchronous appressorial formation.

The variability within experiments was less with lower concentrations of sucrose, but increased with increasing sucrose concentrations (Table 1). The percentage variability at the 1 percent level of sucrose was 4 percent.

There was no appreciable change in the percentage of conidia forming appressoria in the control experiments with different batches of conidia.

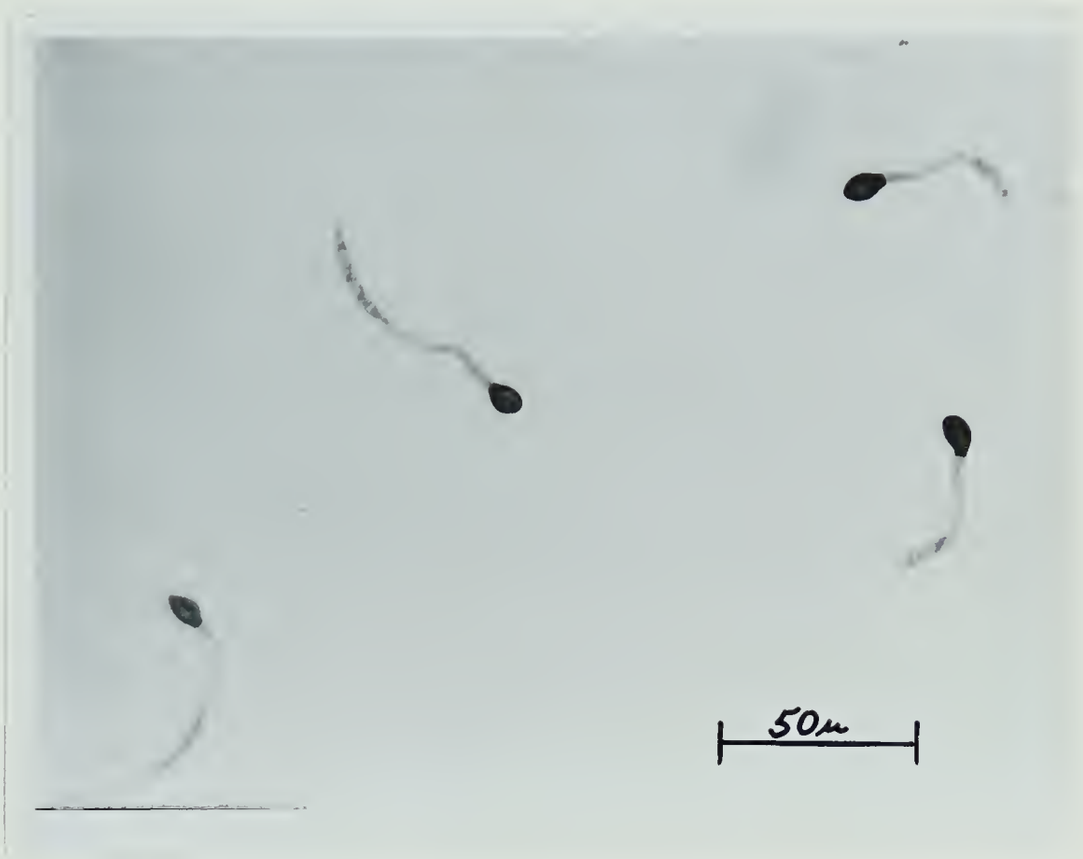


FIG. 1. Appressoria of C. graminicola formed in a 1 percent sucrose solution on a glass micro slide.

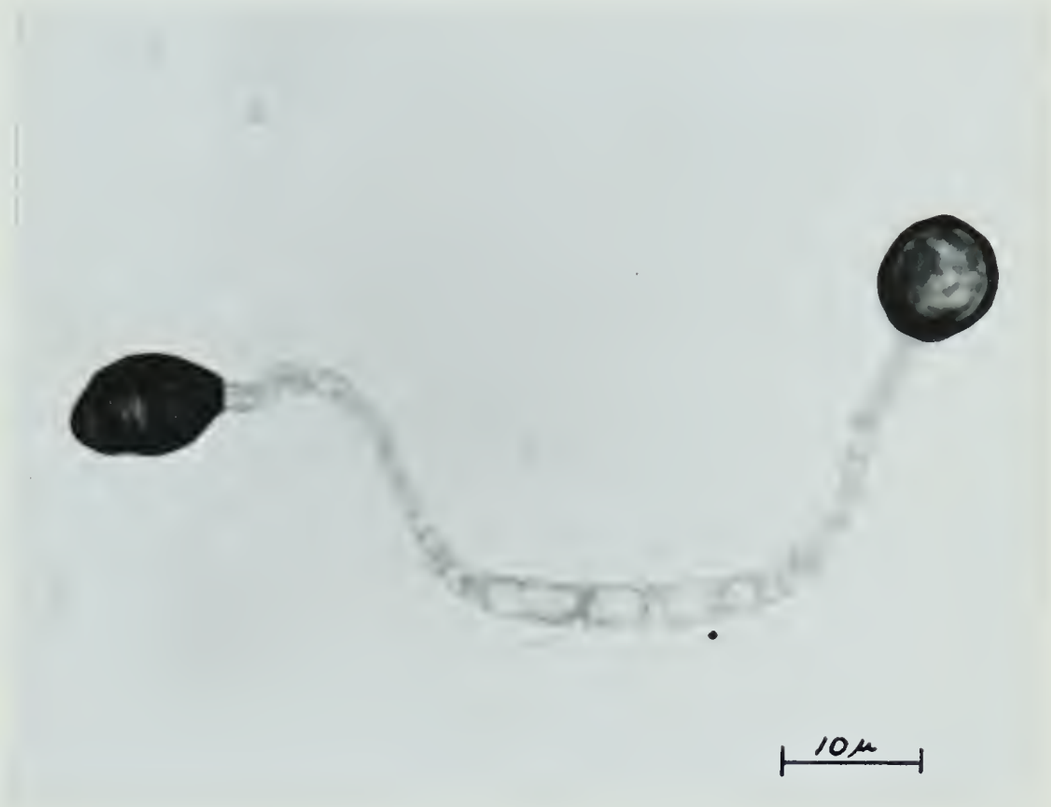


FIG. 2. Typical appressoria formed at the ends of germination tubes arising from a conidium of C. graminicola.

Table 1. The effect of different concentrations of sucrose on germination of conidia and on production of appressoria in C. graminicola

Sucrose Concentration (%)	Conidia Germinating (%)	Conidia Producing Appressoria (%)	Standard Deviation [1] (%)
0	3	94	2
0.1	25	94	2
0.5	45	92	3
1.0	98	92	4
5.0	98	88	4
10.0	98	83	7
20.0	95	63	5
30.0	96	58	7
40.0	NG[2]	NG[2]	-

[1]Standard deviation of conidia producing appressoria.
[2]No germination.

(ii) Carbohydrates

Amygdalin was the only carbohydrate that inhibited appressorial formation below the 50 percent level of germinating conidia, while, in the remaining 19 carbohydrates, over 60 percent of the germinating conidia produced appressoria (Fig. 3). The percentage of conidia that germinated was high in all media, except in some higher concentrations of benzaldehyde and mandelonitrile.

Benzaldehyde and mandelonitrile, the breakdown products of amygdalin, completely inhibited germination of conidia at a concentration of 0.1 percent, but did not show any inhibitory effect on

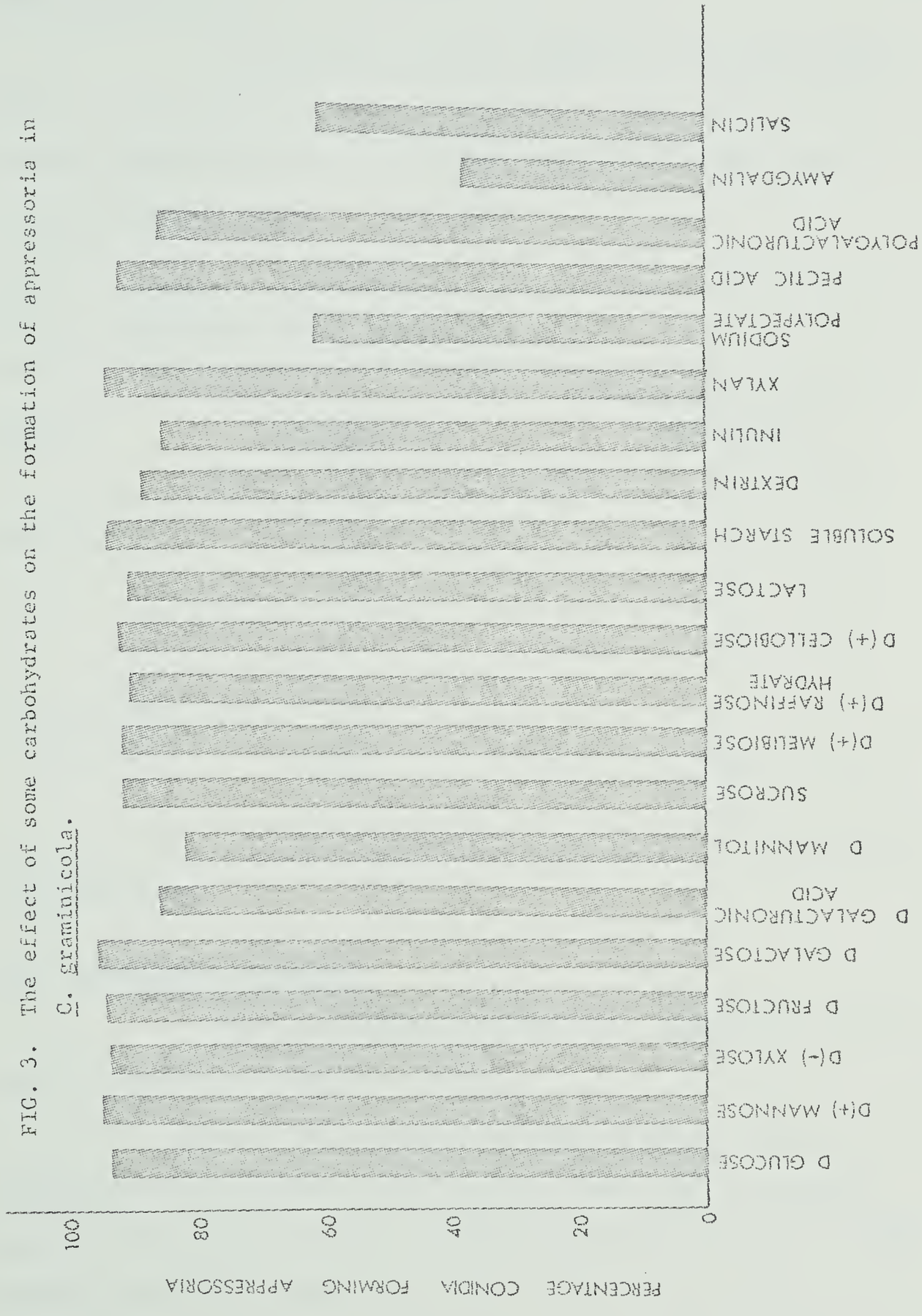


FIG. 3. The effect of some carbohydrates on the formation of appressoria in C. graminicola.

germination and appressorial formation at concentrations of 0.01 and 0.001 percent (Table 2).

In experiments involving amygdalin as a carbon source, HCN was detected after 24 hours of incubation. The sodium picrate turned a reddish-brown color indicating the release of HCN, while controls remained yellow.

Table 2. Effect of benzaldehyde and mandelonitrile on production of appressoria in C. graminicola

Chemical	Concentration (%)	Conidia Producing Appressoria (%)
Benzaldehyde	0.001	95
	0.01	93
	0.1	0*
Mandelonitrile	0.001	93
	0.01	91
	0.1	0*

*No germination

(iii) Nitrogenous Compounds

A high percentage of conidia of C. graminicola germinated in each of the nitrogenous media.

None of the inorganic nitrogen compounds inhibited appressorial formation below the 50 percent level and, in ammonium phosphate and ammonium sulfate approximately 90 percent of the conidia produced appressoria (Table 3).

Table 3. The effect of inorganic nitrogen compounds on the formation of appressoria in C. graminicola

Chemical	Conidia Forming Appressoria (%)
Ammonium phosphate	87
Ammonium sulfate	90
Ammonium nitrate	56
Potassium nitrate	53
Potassium nitrite	52

Over 80 percent of the conidia produced appressoria in each of the amino acid solutions, except in cysteine, which reduced appressorial formation to approximately the 1 percent level (Table 4).

Table 4. The effect of amino acids on the formation of appressoria in C. graminicola

Amino Acid	Conidia Forming Appressoria (%)
Glycine	95
Alanine	96
Serine	91
Valine	98
Leucine	87
Aspartic acid	98
Citrulline	96
Asparagine	86
Cystine	94
Cysteine HCl	1
Tyrosine	94
Methionine	93
Phenylalanine	84
Proline	96
Histidine	96

None of the peptides exhibited any marked inhibition on appressorial formation in C. graminicola (Table 5).

Table 5. The effect of peptides on appressorial formation in C. graminicola

Peptide	Conidia Forming Appressoria (%)
glycyl L tyrosine	75
DL alanyl DL valine	94
glycyl DL aspartic acid	85
DL alanyl DL alanine	93
L phenylalanine DL alanine	92
glycyl DL serine	90
DL alanyl DL leucine	88
glycyl L proline	83
glycyl D asparagine	92

In each of the following peptone solutions, used at a concentration of 1 percent, less than 1 percent of the conidia of C. graminicola produced appressoria: Soy Peptone Bacteriogical Bacto-Peptone, Bacto-Soytone, Soy Hydrolysate, Proteose-Peptone and Peptone-S. Soy Peptone Bacteriological, at concentrations of 0.1, 0.01 or 0.001 percent, used with or without sucrose, however, resulted in over 85 percent of conidia producing appressoria.

Proteins varied considerably in their effect on appressorial formation in C. graminicola (Table 6). A highly inhibitory effect occurred in trypsin, inactivated trypsin, crude

protease, peroxidase, pronase, polyphenol oxidase and cytochrome C oxidoreductase. The remaining protein compounds did not show any appreciable inhibition of appressorial formation.

Table 6. The effect of proteins on appressorial formation in *C. graminicola*

Protein	Concentration (%)	Percentage of Conidia Producing Appressoria
Trypsin	0.1	1
	0.01	11
	0.005	46
Trypsin (inactivated)	0.1	25
Crude protease	0.1	1
Peroxidase	0.2	0
	0.1	9
Pronase	0.1	0
Alpha amylase	0.1	87
Lipase	0.1	90
Cellulase	0.1	79
Catalase	0.1	93
Polyphenol oxidase	0.1	31
Glucose oxidase	0.1	92
Cytochrome C oxidoreductase	0.1	31
Pectinase	0.1	94

(iv) Fatty Acids

The following fatty acids did not inhibit conidial germination or appressorial formation in C. graminicola (percentage of conidia forming appressoria is listed in brackets for each compound): stearic acid (96), palmitic acid (94), oleic acid (94) and linoleic acid (92).

(v) Sulfur-containing and Related Compounds

The inorganic forms of sulfur, K_2SO_4 and $K_2S_2O_5$, in a 0.1 percent solution, had no inhibitory effect on appressorial formation.

The sulfur-containing compounds with sulphydryl (-SH) groups (cysteine, homocysteine, thiohistidine, glutathione and 2-mercaptoethylamine) were highly inhibitory to appressorial formation when used in concentrations of 0.5 and 1.0 percent, while those having oxidized -SH groups (cysteine sulfinic acid, cysteic acid, homocysteic acid and oxidized glutathione) and S-methyl cysteine did not have any appreciable adverse effect (Table 7). At the lower concentration of 0.1 percent, the reduced forms, with the exception of homocysteine and 2-mercaptoethylamine, did not inhibit appressorial formation.

Penicillin G, a sulfur-containing antibiotic, strongly inhibited appressorial formation at concentrations of 100,000 and 50,000 I.U.'s, but had no adverse effect at a lower concentration of 10,000 I.U.'s. The percentages of conidia producing appressoria in decreasing concentrations of penicillin were 1, 20 and 91.

Potassium tellurite inhibited appressorial formation in a 0.002 M solution but had no adverse effect at a concentration of 0.0002 M. Potassium selenite was highly inhibitory to appressorial formation only at a concentration of 0.02 M.

Table 7. The effect of sulfur-containing and related compounds on appressorial formation in C. graminicola

Chemical	Percentage of Conidia Producing Appressoria in Chemical Concentrations					
	0.1%	0.5%	1.0%	0.02M	0.002M	0.0002M
K_2SO_4	97	NG*	NG*			
$K_2S_2O_5$	96	NG*	NG*			
Cysteine	89	5	1			
Cysteine sulfinic acid	94	93	89			
Cysteic acid	94	96	95			
Homocysteine	36	5	6			
Homocysteic acid	97	96	97			
Thiohistidine	92	1	1			
S-methyl cysteine	95	90	97			
Glutathione	86	57	4			
Glutathione (oxidized)	94	91	83			
Methionine sulfone	97	90	96			
Methionine sulfoxide	97	95	97			
Mercaptoethylamine	5	0	NG*			
Potassium tellurite	NG*			NG*	15	85
Potassium selenite	NG*			29	95	97

*No germination

(vi) Carbon:nitrogen Ratios

Inorganic salts as nitrogen sources

There was a marked inhibitory effect on appressorial formation in each concentration of salt (except NH_4NO_3) at the 0.5 percent or higher levels, but in each concentration of each salt the effect on appressorial formation was relatively uniform throughout the sucrose concentrations (Table 8). NH_4NO_3 also had an inhibitory effect in all sucrose concentrations when used at levels of 1.0 percent or higher.

Table 8. The effect of different ratios of carbon:nitrogen, with inorganic sources of nitrogen, on appressorial formation in C. graminicola

Nitrogenous Salt	Concentration (%)	Percentage appressorial formation in sucrose concentrations (%)			
		0.1	0.5	1.0	5.0
KNO_3	0.05	87	91	94	89
	0.1	96	93	88	92
	0.5	50	66	67	63
	1.0	59	53	51	49
	2.0	15	3	13	7
KNO_2	0.05	93	95	90	94
	0.1	92	92	92	95
	0.5	43	41	55	50
	1.0	49	45	45	55
	2.0	NG*	NG*	NG*	NG*
NH_4NO_3	0.05	95	98	94	96
	0.1	96	98	98	97
	0.5	71	86	69	86
	1.0	52	34	51	32
	2.0	26	26	21	9
NH_4NO_2	0.05	93	95	97	95
	0.1	93	97	79	85
	0.5	47	44	19	19
	1.0	17	9	9	8
	2.0	8	11	5	4

*No germination

In most combinations, except with KNO_2, the inhibitory effect increased with each increase in the concentration of salt.

Amino acids as nitrogen sources

Cysteine was highly inhibitory to appressorial formation at the 0.5 and 1.0 percent levels in all concentrations of sucrose, while the remaining amino acids did not show any adverse effect on appressorial formation in any concentration of sucrose (Table 9).

Table 9. The effect of different ratios of carbon:nitrogen, with amino acids as nitrogen sources, on appressorial formation in *C. graminicola*

Amino Acid	Concentration (%)	Percentage appressorial formation in sucrose concentrations (%)			
		0.1	0.5	1.0	5.0
Glycine	0.1	97	94	91	96
	0.5	95	95	97	93
	1.0	88	88	97	91
Alanine	0.1	95	97	94	94
	0.5	95	95	95	95
	1.0	94	93	93	94
Serine	0.1	94	93	94	96
	0.5	93	96	95	94
	1.0	94	96	94	94
Cysteine	0.1	83	93	89	86
	0.5	2	2	5	6
	1.0	1	1	1	3
Threonine	0.1	93	96	96	94
	0.5	95	94	96	95
	1.0	95	95	96	95

Table 9 Continued

Amino Acid	Concentration (%)	Percentage appressorial formation in sucrose concentrations (%)			
		0.1	0.5	1.0	5.0
Methionine	0.1	96	94	96	97
	0.5	90	95	95	94
	1.0	97	92	95	97
Leucine	0.1	97	96	98	97
	0.5	95	94	94	94
	1.0	94	94	LG*	LG*
Glutamic acid	0.1	97	96	96	97
	0.5	92	94	93	94
	1.0	96	96	94	92
Arginine	0.1	94	96	98	96
	0.5	98	91	94	95
	1.0	93	91	91	92
Lysine	0.1	84	88	81	81
	0.5	93	91	91	92
	1.0	LG*	LG*	LG*	LG*
Phenylalanine	0.1	97	96	95	95
	0.5	97	95	94	94
	1.0	96	94	96	94
Histidine	0.1	96	97	97	97
	0.5	95	96	95	94
	1.0	91	92	96	95
Proline	0.1	96	92	96	94
	0.5	96	95	96	96
	1.0	96	96	94	92

* Low level of germination

None of the peptides, used in combination with sucrose, had any inhibitory effect on appressorial formation in C. graminicola (Table 10).

Table 10. The effect of different ratios of carbon:nitrogen, with peptides as nitrogen sources, on appressorial formation in C. graminicola

Peptide	Concentration (%)	Percentage appressorial formation in sucrose concentrations (%)			
		0.1	0.5	1.0	5.0
DL alanyl DL methionine	0.1	94	97	94	94
	0.5	94	90	96	92
	1.0	92	91	92	96
Glycyl DL serine	0.1	98	96	96	96
	0.5	98	94	96	94
	1.0	97	97	96	93
Glycyl DL leucine	0.1	97	96	96	96
	0.5	95	96	98	97
	1.0	93	94	94	93
Alanyl DL phenylalanine	0.1	95	94	94	95
	0.5	93	93	96	92
	1.0	96	94	94	93
DL alanyl glycine	0.1	98	95	96	94
	0.5	95	93	95	94
	1.0	96	95	95	93

Peptone S and Soy Peptone Bacteriological, in 0.5 or 1.0 percent solution, almost completely inhibited appressorial formation but had no adverse effect at a concentration of 0.1 percent, while Proteose-Peptone almost completely inhibited appressorial formation at 0.5 and 1.0 percent, and at 0.1 percent inhibited appressorial formation below the 50 percent level (Table 11).

Table 11. The effect of different ratios of carbon:nitrogen, with peptones as nitrogen sources, on appressorial formation in C. graminicola

Peptone	Concentration (%)	Percentage appressorial formation in sucrose concentrations (%)			
		0.1	0.5	1.0	5.0
Peptone S	0.1	92	90	94	92
	0.5	2	3	2	2
	1.0	1	1	1	0
Soy Peptone Bacteriological	0.1	84	78	86	95
	0.5	2	2	1	1
	1.0	1	0	0	0
Proteose-Peptone	0.1	43	38	27	45
	0.5	4	1	2	0
	1.0	1	0	1	0

(vii) Cysteine combined with other nitrogenous sources

Fifteen different nitrogenous compounds, used singly in combination with L-cysteine HCl, did not alter the highly inhibitory effect of cysteine on appressorial formation. In all combinations with cysteine the percentage of conidia of C. graminicola that produced appressoria ranged from 0 to 4.

II. Hydrogen-ion Concentration

There was no inhibitory effect on appressorial formation in the pH range of 3-9, and only a slight amount of inhibition occurred at pH's of 10 and 11 (Fig. 4). Conidia did not germinate at pH's of 1.5, 2 and 12.

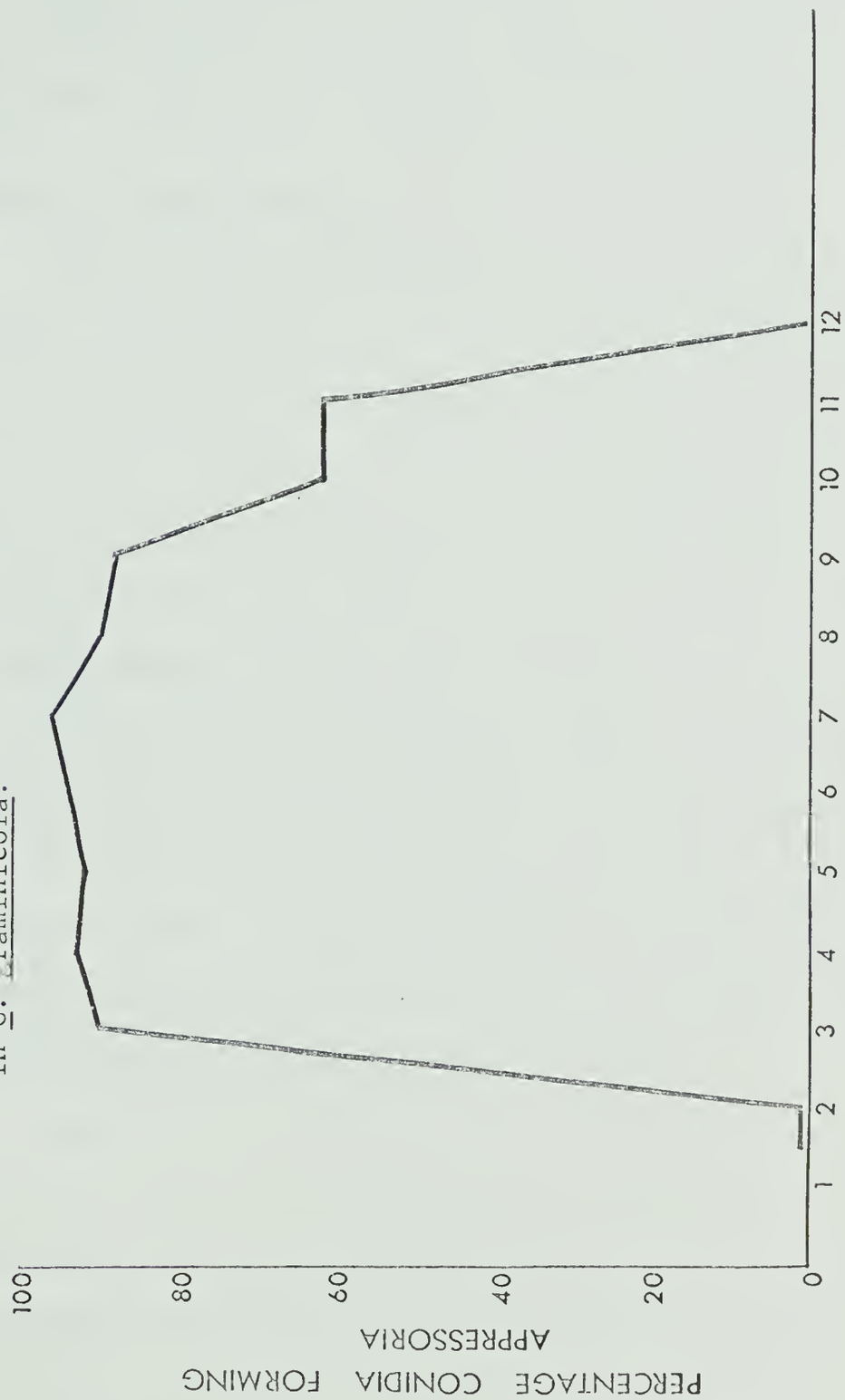


FIG. 4. The effect of hydrogen-ion concentration on appressorial formation in C. graminicola.

III. Physical Factors

(i) Hanging Drop

Appressoria were formed in hanging drop preparations only at points where the germination tubes were in contact with the glass slide. Conidia which germinated on the lower surface of the hanging drop, away from the glass slide, did not produce appressoria.

(ii) Membranes of Graded Hardness

Formalized gelatin membranes of graded hardness, placed on glass slides or on 2 percent water agar, differentially influenced appressorial formation. After 24 hours of incubation in a 1 percent sucrose solution, some appressoria were observed on the harder membranes prepared in 60 or 80 percent ethanol, but none appeared on the softer membranes prepared in 0, 20, or 40 percent ethanol. After 48 hours of incubation appressoria were formed abundantly and in largest numbers on the 80 percent ethanol membranes, (Fig. 5A), in intermediate amounts on the 60 percent ethanol membranes, and only in trace amounts on membranes prepared in 40 percent ethanol. Appressoria did not form on 0, or 20 percent ethanol membranes (Fig. 5B). However, appressoria formed abundantly on membranes prepared in 0 and 20 percent ethanol after they were hardened by partial drying.

Appressorial formation was completely inhibited on all membranes when Soy Peptone Bacteriological, at a concentration of 1 percent, was included in the medium containing conidia in suspension. There was an extensive development of mycelium in this medium.

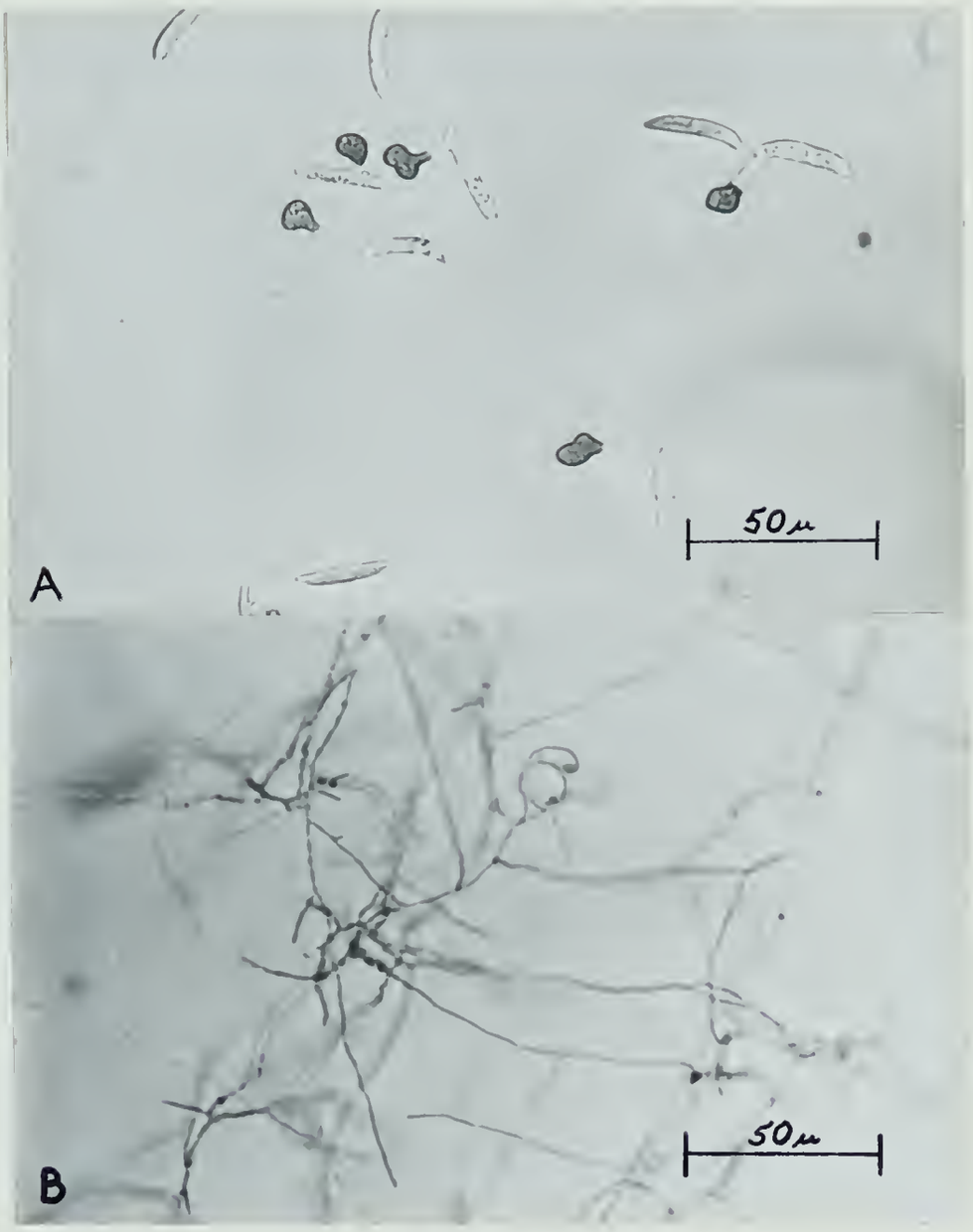


FIG. 5. Growth of C. graminicola on gelatin membranes of graded hardness.

A. Hardest membrane, showing excellent appressorial formation.
B. Softest membrane, showing exclusively mycelial growth.

(iii) Glass Wool

Mycelium of C. graminicola, on 2 percent water agar, grew sparsely on the surface and to a less extent within the agar. Appressoria formed almost exclusively at points where hyphal tips came in contact with glass wool on the surface or embedded in agar.

In 2 percent water agar, supplemented with Soy Peptone Bacteriological at a concentration of 1 percent, the amount of mycelial growth was considerably greater than in water agar alone, but appressorial production occurred in only trace amounts. In this medium appressoria were also formed almost exclusively at points of mycelial contact with glass wool (Fig. 6).

(iv) Excised Leaves

Over 80 percent of the appressoria were observed at junctions of epidermal cells of barley leaves.

B. Factors Affecting Function of Appressoria

I. Penetration

Entrance into the leaf was accomplished by means of a fine penetration peg that developed on the underside of an appressorium. Penetration occurred directly through the cuticle and epidermal cell wall. The cell wall appeared to swell in some cases even before penetration had begun. In many cases a thickening of the cell wall beneath the appressorium resulted in the formation of a callosity (Fig. 7 A, B, C, D). The penetration peg was very fine in the cuticle and enlarged as it entered the cell wall. The infection hypha in the callosity enlarged further before passing through the cell wall (Fig. 7 D), but

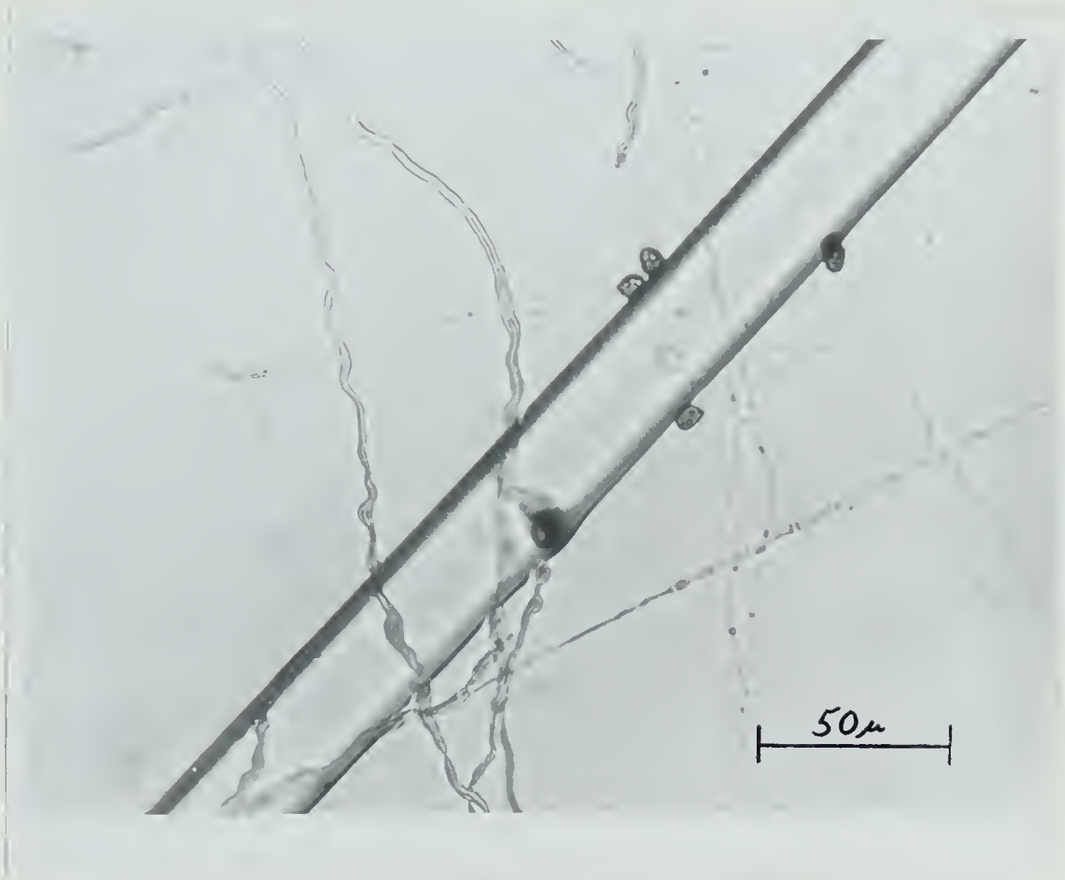


FIG. 6. Formation of appressoria in C. graminicola at points of mycelial contact with glass wool embedded in water agar.

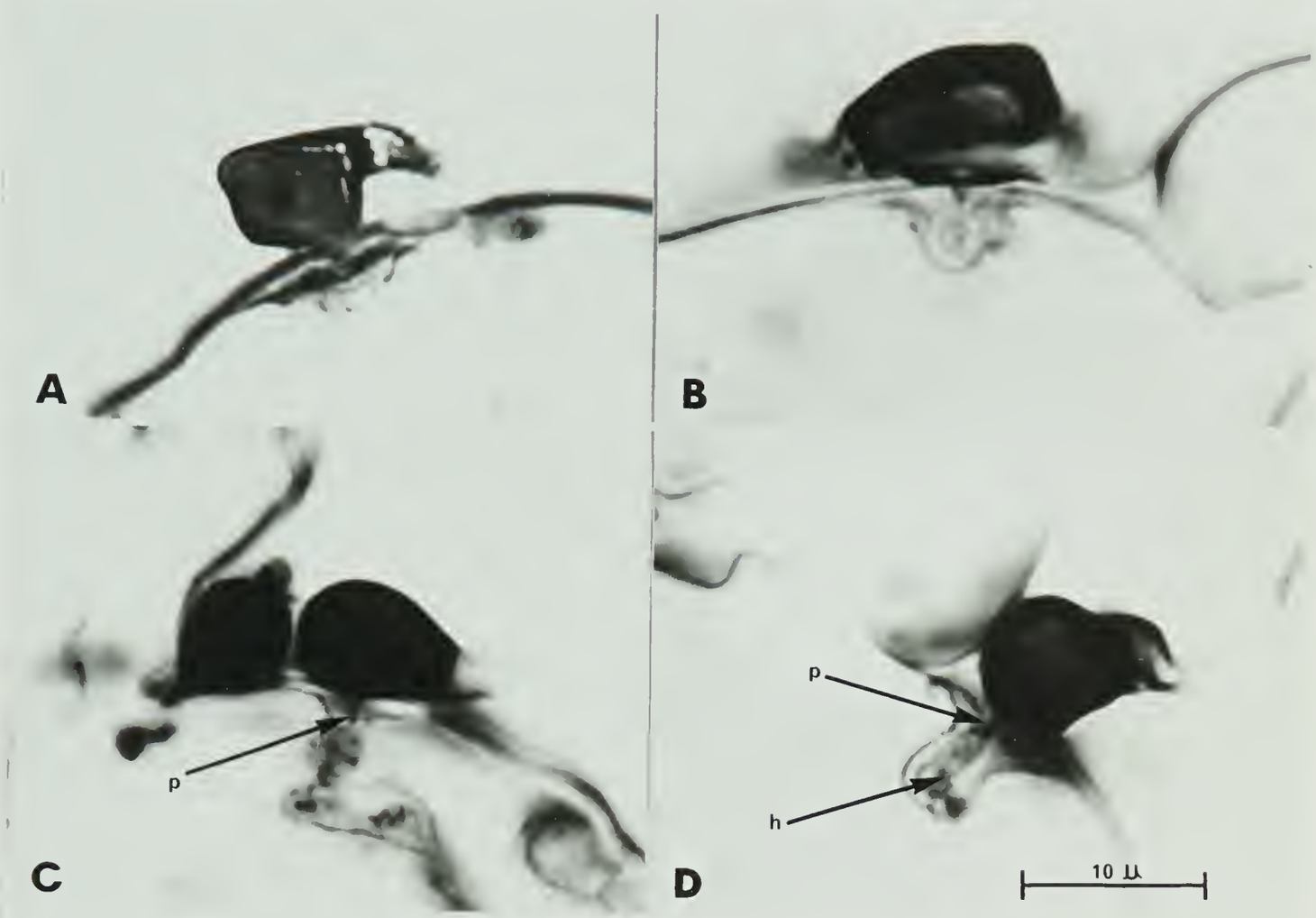


FIG. 7. Cross-sections of barley epidermis showing the fine details of penetration by C. graminicola.

A. A thickening of the epidermal cell wall directly beneath the appressorium.
B. A penetration peg appears and thickening of epidermal cell wall increases.
C. Penetration completed, showing penetration peg (p) and hypha of normal diameter in cell lumen.
D. Well developed callosity showing penetration peg (p) and enlarged infective hypha (h).

resumed its normal diameter on entering the cell lumen (Fig. 7 C).

The first leaf of Moore barley showed a pattern of initial susceptibility to penetration, followed by a period of resistance, and then susceptibility again, regardless of the light intensity in which it was grown (Fig. 8). During the first 6 days the leaf was highly susceptible to penetration for each light intensity, but the period of resistance was progressively shorter with each increase of light intensity. Thus, leaves from plants grown in a light intensity of 1200 foot-candles were resistant to penetration for approximately 15 days, those grown at 1600 foot-candles remained resistant for approximately 9 days, and those grown at 2200 foot-candles remained resistant for only 6 days.

The third leaf of Moore barley, grown in a light intensity of 1600 foot-candles, was also readily penetrated during the first 6 days, but then became resistant for 9 days (Fig. 9).

In experiments involving the effect of temperature on penetration of the third leaf during its period of resistance, none of the leaves showed obvious evidence of yellowing during the 5-day period of the experiment. Leaves, which were held at 20° or 30° C for 48 hours prior to inoculation with conidia of C. graminicola, remained resistant to penetration when incubated for 72 hours at 20° C. However, leaves which were pretreated at 30° C for 48 hours prior to inoculation, were readily penetrated within 72 hours when incubated at 30° C.

FIG. 8. The effect of aging and light intensity on the penetration of the first leaf of barley by C. graminicola.

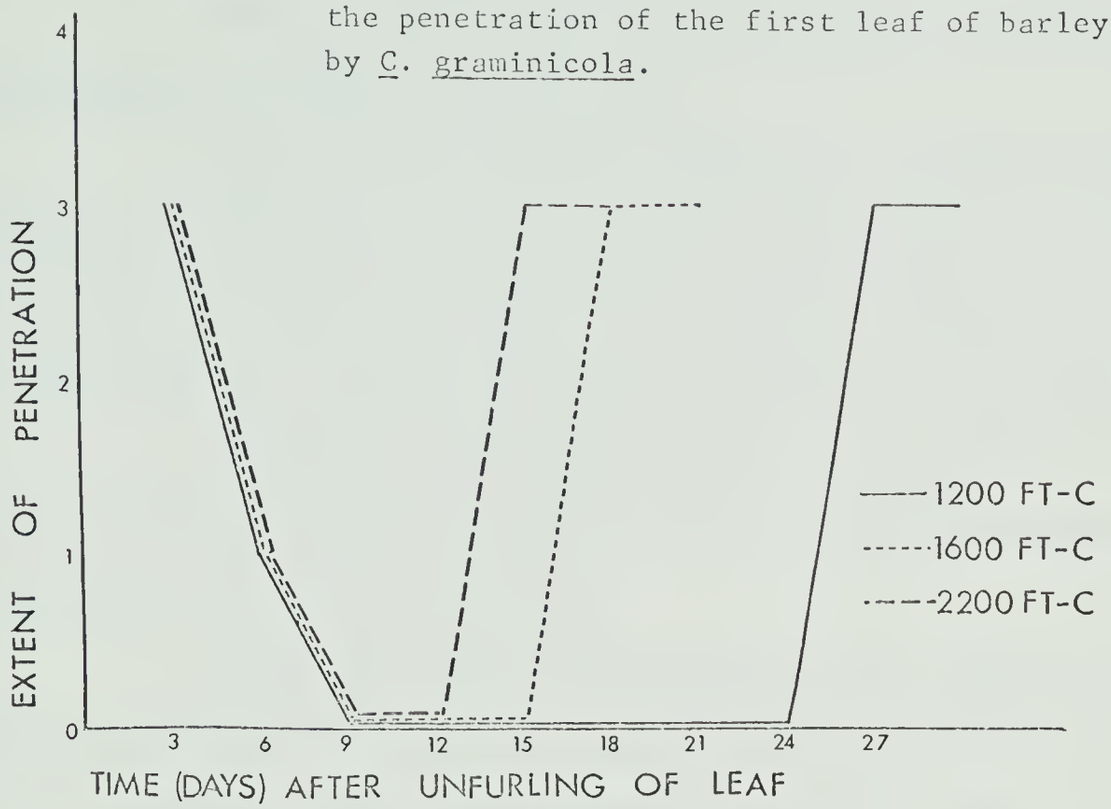
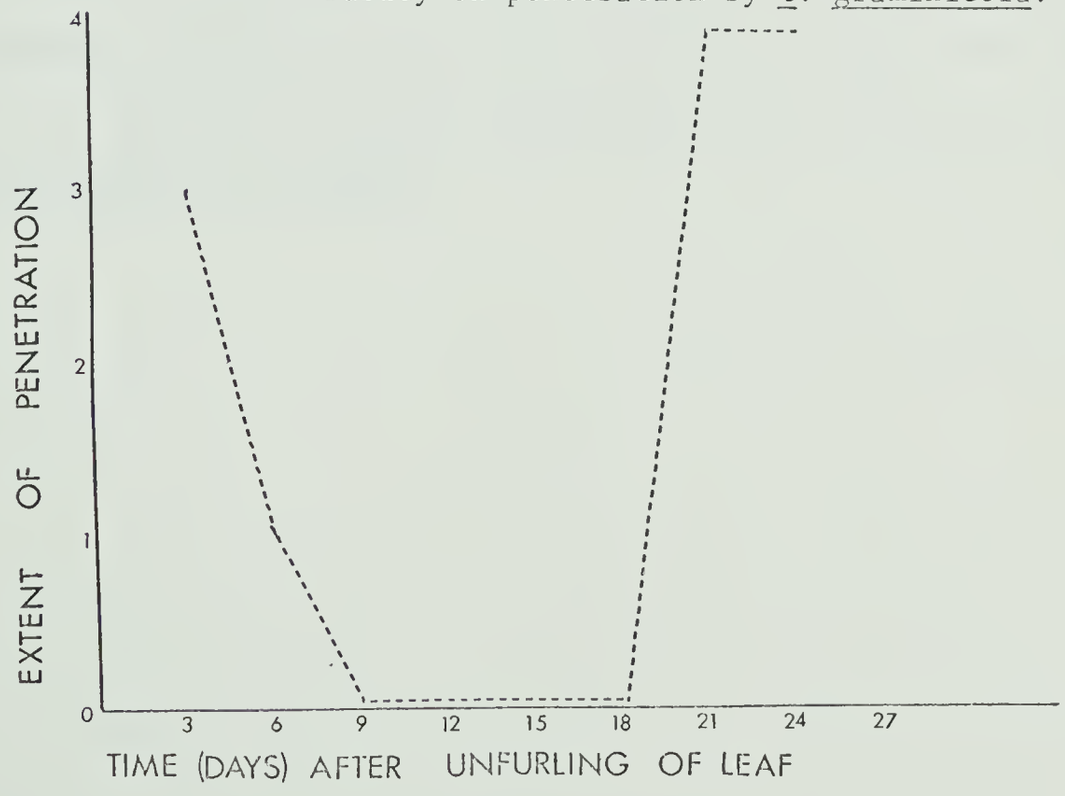


FIG. 9. The effect of aging of the third leaf of barley on penetration by C. graminicola.



II. Survival

Conidia which were air-dried immediately following harvesting failed to germinate after 1 day.

Mycelium produced from conidia in a 1 percent sucrose solution to which Soy Peptone Bacteriological was added at a concentration of 2 percent, remained viable for approximately 400 days in an air-dried condition. Appressoria were not produced in this medium.

Appressoria, which were produced in a 1 percent sucrose solution and which remained attached to the cover slips, have now retained their viability for 500 days.

Appressoria which were detached from the glass surface and air-dried immediately, failed to germinate when seeded on PSA. Detached appressoria, which were seeded on PSA without prior air-drying, germinated readily. Detached appressoria, which were not dried and were used to inoculate barley leaves immediately, penetrated by producing a penetration peg in most instances. A small percentage of these appressoria produced a germination tube at the end of which an appressorium was formed.

DISCUSSION

C. graminicola possesses several characteristics which make it a suitable fungus for investigations concerning factors affecting appressorial formation. It produces relatively large, dark appressoria within 24 hours in a variety of nutrients and over a wide range of temperatures. These appressoria usually arise at the tips of short germination tubes of the conidia in artificial culture or on the plant surface, thus making it is easy to determine their source.

Since there is a scarcity of information in literature on the effect of nutrition on appressorial formation, an emperical approach to the choice of nutrients to be used in this study was necessary. They were selected primarily on the basis of their prevalence in plant material or on their chemical relationship to compounds that may be found in plants. Nutrients that exhibited a strong inhibitory effect on appressorial formation were given special attention in an attempt to explain their possible mode of action.

A 1.0 percent sucrose solution was chosen as a basal medium because it induced an almost complete and synchronous germination of conidia. These prerequisites were desirable because germination that extended over a long or irregular time interval could cause inaccuracy in tabulation of appressorial formation.

A common characteristic of most nutrient compounds that strongly inhibited appressorial formation was the presence of sulphydryl (-SH) groups in their structures, or the potential to form this group. The -SH-containing amino acids, the tripeptide glutathione and 2-mercaptoethylamine were all highly inhibitory to appressorial formation.

However, oxidized forms of cysteine and oxidized glutathione did not show any inhibition. The inhibitory effect of appressorial formation was lost when the hydrogen on the sulfur of cysteine was replaced by a methyl group, e.g., S-methyl cysteine.

Other sulfur-containing amino acids, cystine, methionine sulfone, methionine sulfoxide and methione, did not inhibit appressorial formation. However, only 0.01 percent cystine was soluble in water and so a direct comparison with cysteine, was not possible. Penicillin, a sulfur-containing antibiotic, and selenium and tellurium, members of the sulfur family, also strongly inhibited appressorial formation.

The enzymatic proteins varied considerably in their effect on appressorial formation. Trypsin, inactivated trypsin, crude proteinase, pronase, peroxidase, polyphenol oxidase and cytochrome C oxidoreductase were highly inhibitory to appressorial formation, while other enzymes did not show this effect.

Since both the active and inactive forms of trypsin were inhibitory to appressorial formation, it appears that the chemical composition and not the configuration of the protein molecule was the determining factor in producing this effect. The mere presence of -SH groups did not appear to be the only factor affecting appressorial formation because some inhibitory and non-inhibitory enzymes contained this group (53, 54). All -SH groups, however, are not necessarily reactive because they may be protected within the protein configuration.

This evidence indicating that -SH content has an important role in inhibiting appressorial formation may possibly be used to explain some of the observations with peptones and proteins. The -SH groups contained in proteins and peptones may act in the same manner as the

-SH groups of -SH-containing amino acids. Denaturation of proteins causes them to uncoil or unfold, resulting in the rupture of cross linkages and consequently increasing the number of active -SH and -S-S- groups.

Amygdalin, a glycoside, used at a concentration of 1.0 percent, was the only carbohydrate that inhibited appressorial formation. It breaks down into benzaldehyde + HCN + glucose, or glucose + mandelonitrile. Glucose, mandelonitrile or benzaldehyde did not inhibit appressorial formation. A direct comparison between amygdalin and its breakdown products, however, was not possible because benzaldehyde and mandelonitrile inhibited conidial germination at a concentration of 1 percent. It was also found that HCN was released during the breakdown of amygdalin. The significance of this observation will be discussed in another portion of this text.

Certain relationships may be made between results obtained in this investigation and information concerning the mode of action of -SH groups in somewhat similar situations. Changes in -SH concentration have long been associated with mitosis and cell differentiation. Hammet and Chapman (20) found that there was a high concentration of -SH groups in meristematic tissue. A high -SH content was associated with mitosis and a low -SH content with cell elongation. Nickerson and Van Rij (39) demonstrated that -SH groups also play an important role in cell division of microorganisms. Chemicals containing -SH groups, such as cysteine and glutathione, promoted cell division and inhibited the appearance of enlarged mycelial structures. Appressoria are developed as enlargements of hyphal tips and thus, their formation may be inhibited through a suppression of this enlargement.

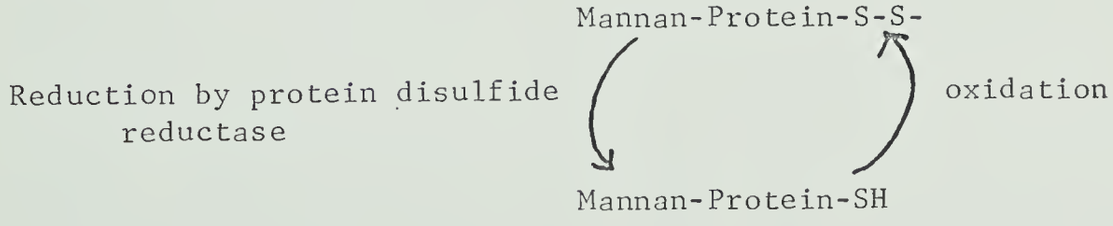
Pratt and Dufrenoy (41) proposed that penicillin, in sub-bacteriostatic concentrations, promoted the conversion of Escherichia coli and Bacillus subtillus cells to a filamentous form. Nickerson and Van Rij (39) made similar observations with yeasts. Pratt and Dufrenoy (41) concluded that penicillin, in sub-bacteriostatic concentrations, stimulated metabolism and increased cell size, but disrupted the process of cellular division. They proposed that penicillin in sub-lethal concentrations affected -SH groups and brought about a shift from -SH to -S-S-groups. In this study, Penicillin G strongly inhibited appressorial formation in C. graminicola even though mycelial growth occurred extensively thus suggesting a possibly similar mechanism.

Selenium and tellurium are members of the sulfur family and selenium is known to be bound to proteins (55) and to be incorporated into organic compounds (40). Nickerson, Taber and Falcone (38) found that selenite and tellurite induced a variety of filamentous microorganisms to grow exclusively in the yeast phase condition. The authors suggested that this was not selective inhibition of filamentous growth, but the promotion of cell division. Nickerson, Taber and Falcone (38) suggested that the protein-bound selenium occurred in the reduced state (SeH) and could not be readily oxidized. They suggested that selenium would affect the function of proteins connected with cell division. Potassium selenite and potassium tellurite were inhibitory to appressorial formation. This suggests a possible interaction of these chemicals and the function of proteins involved in appressorial formation.

Robson and Stockley (45) used autoradiography to locate and follow changes in -SH concentrations in fungi. Autoradiographs of Candida albicans and Eremothecium ashbyii showed that -SH groups were localized in specific areas of the cell wall of these two fungi. The -SH reaction for these two fungi was very strong in the areas where hyphal buds were formed. In Aspergillis niger and Penicillium chrysogenum -SH groups were present in the cell walls and cytoplasm.

Nickerson (36) proposed a scheme to explain the budding mechanism of yeasts involving the interaction of -SH and -S-S- groups of the polysaccharide-protein complex. There are two types of yeasts: the so-called, normal yeasts which reproduce by budding, and the filamentous or divisionless yeasts. Nickerson and Falcone (37) found that a protein disulfide reductase reduced the -S-S- links of the glucomannan-protein. This disulfide reductase was present in the yeasts that reproduced by budding, but occurred only in trace amounts in the divisionless strains. Nickerson (35) reported that -SH donors, such as cysteine, maintained spherical growth of yeast cells and in another study he and Van Rij (39) showed that, when cysteine was added to the growth media, the divisionless yeasts resumed a budding type of reproduction.

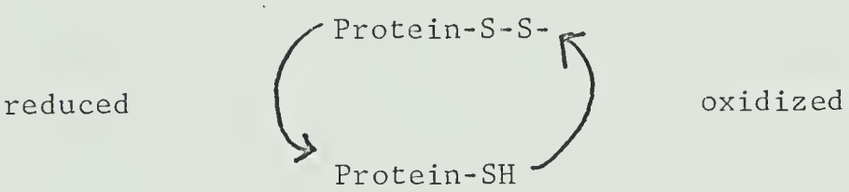
Nickerson (36) proposed the following scheme for the operation of the protein disulfide reductase in the budding process of yeasts.



The cell wall is elastic when the sulfur is covalently bonded and thus linear extension can occur. If the mannan-protein-S-S- is reduced to mannan-protein-SH, the cell wall can undergo plastic deformation and the fibrillar ordering necessary for cell division. The orientation of macro-molecular components of the cell wall when examined in electron micrographs, show a random distribution of micro-fibrils. This is expected with covalent bonding of the -S-S- groups and was found to be the case in all parts of the yeast cell wall except in the area of bud formation. At the site of bud formation there was a circular ordering of fibrillar components of the yeast cell wall.

In this study, compounds containing -SH groups, cysteine, homocysteine, thiohistidine, glutathione and 2-mercaptoethylamine, may have inhibited appressorial formation by preventing the swelling of the hyphal tip which is the first stage in the formation of an appressorium.

Another mode of action of -SH groups in inhibiting appressorial formation is that proposed by Nickerson (36).



Protein, containing sulfur in -S-S- bonds, would allow for linear extension of mycelium, but would not allow the swelling of hyphal tips. Protein with -SH groups would allow for the plastic deformation necessary for appressorial formation. An excess of -SH groups, supplied externally, may act by slowing down or preventing the forward reaction from -S-S- $\longrightarrow$ -SH. The reverse reaction may be stimulated with the net result that -S-S- bonds would be

maintained, thus preventing appressorial formation.

The oxidation and reduction of -SH and -S-S- linkages are of prime importance in enzyme activity. According to Boyer (4) -S-S- linkages are cleaved by -SH-containing compounds such as glutathione. This may result in changes in the reactivity of enzymes containing -S-S- groups. The cleavage of the -S-S- bond to form -SH groups may result in the activation of enzymes by forming -SH groups essential for enzyme activity. The cleavage of -S-S- bonds may result in inactivation of the enzyme presumably by altering the structure of the enzyme. Thus, the effect of -SH-containing compounds could bring about inhibition of appressorial formation by affecting an enzyme or enzymes associated with appressorial formation.

The presence of HCN during the breakdown of amygdalin may influence appressorial formation through it's role in the production of -SH groups. Irving et al (24) observed that HCN activated proteolytic enzymes. Frankel-Conrat (16) showed that HCN may cause hydrocyanolysis of -S-S- groups to form -SH groups in the following reaction: R-S-S-R + HCN $\longrightarrow$ R-SH + R-S-CN. However, Frankel-Conrat could not decide if this effect could be used to explain the activating role of HCN on enzymes. Thus, it is possible that HCN may activate enzymes which result in the inhibition of appressorial formation.

Another completely different explanation may be advanced to explain the effect of -SH groups on appressorial formation. Sulphydryl groups may influence appressorial formation by affecting membrane permeability. Robinson (44) reported that changes in -SH content of the membrane will alter its permeability. There is a close association

between the lipid and protein in membranes, but interactions are poorly understood. Interactions may occur between protein -SH bonds and the lipid double bonds. Robinson concluded that the addition of -SH reagents would affect the lipid protein association and thus alter the permeability of the membrane.

It is generally accepted that the proper balance among constituents of the culture medium is important in the growth and development of fungi. It has been demonstrated that the carbon:nitrogen ratio of the medium can affect growth, and the formation of reproductive structures (reviewed by Hawker (22)). There was no correlation between the C:N ratios and appressorial formation. When used as nitrogen sources of C:N ratios, inorganic salts did not show any evidence of being a factor in inhibiting appressorial formation. The reduction in appressorial formation increased with each additional increase in concentration of nitrogen, but this occurred with all concentrations of sucrose and thus, the ratio in which the two nutrients were present was of no consequence.

Amino acids, with the exception of cysteine, employed as nitrogen sources for C:N ratios, were not inhibitory to appressorial formation. Cysteine inhibited appressorial formation at 0.5 and 1.0 percent levels, regardless of the level of carbon as sucrose. There was no evidence that C:N ratios were involved in the inhibition of appressoria with any of the C:N ratios used.

Peptones, as a source of nitrogen for C:N ratios, were all strongly inhibitory to appressorial formation at 0.5 and 1.0 percent levels. Proteose-Peptone also inhibited appressorial formation at the

0.1 percent level. Proteose-Peptone contains the reaction products of the protein and the enzyme protease. Proteose-Peptone was inhibitory at 0.1 percent level probably because protease alone was inhibitory at this level.

Fifteen nitrogenous compounds, used singly, were tested in combination with cysteine to determine if other nitrogen sources would alter the inhibitory effect of cysteine on appressorial formation. The addition of other nitrogen compounds did not alter the effect of cysteine indicating that inhibition was not caused by an unavailability of nitrogen, but most likely by the presence of a -SH group.

The physical factors which affected appressorial formation in C. graminicola involved contact with surfaces of different degrees of hardness as well as the interaction of the contact stimulus and nutrition. In hanging drop preparations appressoria were formed only where germ tubes or mycelium came in contact with the glass slide. Conidia, floating on the lower side of the hanging drop, germinated but did not form appressoria. These results confirmed the findings of De Bary (10), Hasselbring (21) and Dey (10) that contact stimulus was required for appressorial formation.

Over 80 percent of appressoria formed over the junctions of epidermal cells on barley leaves. It had been observed by De Bary (10), Hasselbring (21), Preece et al (42) and Meredith (34), that appressoria formed preferentially at this location. There is a slight depression at the junction between epidermal cells, and germ tubes growing into this depression encounter an obstacle. Thus, appressoria were probably formed at this location in response to a contact stimulus.

The contact stimulus for appressorial formation in C. graminicola is influenced by the hardness of the membrane and by the available nutrients. Gelatin membranes of varying degrees of hardness differentially affected appressorial formation. Appressoria were not observed on the two softest membranes, although there was good mycelial growth, while the hardest membrane stimulated excellent appressorial formation, but no mycelial growth. Conidia produced mycelium abundantly in a 1 percent Soy Peptone Bacteriological medium placed on these membranes, but appressoria were not formed even on the hardest membranes. This result is in agreement with Van Burgh's (52) experiments with Colletotrichum phomoides on gelatin membranes. She also found that certain nutrients added to the membranes, prevented appressorial formation. The nutrients were not listed and, therefore, a direct comparison is not possible. It would thus appear that both hardness and nutrition are factors which control appressorial formation.

Experiments involving glass wool embedded in water agar also indicate that both hardness and nutrition are involved in appressorial formation. Appressoria formed almost exclusively at points of contact of hyphal tips with glass wool. Contact with the agar did not stimulate appressorial formation because of its relatively soft consistency. When 1 percent Soy Peptone Bacteriological was added to the water agar, appressorial formation was inhibited even though hyphal tips came in contact with the glass wool.

Thus, both the surface contacted and nutrition are important factors in appressorial formation. Contact is important in stimulating appressorial formation, but the contact stimulus appears to function only if the membrane or object contacted is of a particular

degree of hardness. Nutrients may inhibit appressorial formation even though the contact stimulus is present. This may be the case within the host where appressoria do not form within invaded host cells, even when hyphae are passing through the cell walls.

Fatty acids, known to be components of plant cuticles, are the some of the first compounds to come in contact with appressoria. Resistance to disease development could, therefore, be expressed at this point through an inhibition of appressorial formation. However, appressoria formed abundantly in each of the fatty acids used in this study.

The fine details of the penetration process were similar to those described by Leech (30) with C. lindemuthianum on beans.

Appressorial formation occurs over a wide range of pH. They were formed abundantly at any pH in which conidia germinated. Considering the wide range of pH's in which appressorial formation was not inhibited, it is unlikely that pH would have any adverse effect on their formation under natural conditions.

The first and third leaves of Moore barley were initially susceptible to penetration, followed by a period of resistance and then susceptibility again. Leaves were initially susceptible in all experiments until approximately 9 days after unfurling. Susceptibility to penetration in the early stages of plant development may be attributed to a poorly developed cuticle. A well developed cuticle has been shown to be responsible for increased resistance to penetration with increasing age (7, 33, 51). This may be the case in the developing barley leaf, but a mechanically tough cuticle alone cannot account for resistance or susceptibility to penetration, since the cuticle is well developed at

maturity and penetration occurs readily at this time. The period of resistance was decreased by increasing the light intensity. With increased light intensity the leaves aged more rapidly, as evidenced by a progression of yellowing, and therefore, the period of resistance was shortened. The leaves became susceptible to penetration after obvious yellowing of the leaves had appeared. These results are in agreement with those of Katsanos and Pappelis (25, 26, 27) who have shown that C. graminicola spread only in areas of dead cells in sorghum stalk tissue.

Appressorial formation and penetration are often considered to respond to the same factors. However, Skoropad (48) demonstrated that C. graminicola formed readily on barley leaves from 15-30° C, but penetration occurred only at the higher temperature range of 25-30° C. It had been suggested that leaves senesced more rapidly at 25 and 30° C and this was a factor in penetration.

The third leaf, used 14 days after it unfurled, was resistant to penetration when incubated at 20° C even though it had been pretreated at 20 and 30° C for 48 hours. However, leaves which were pretreated at 20° C for 48 hours and then incubated at 30° C were penetrated readily. It would thus appear that senscence that occurred while the leaf was being pretreated at various temperatures did not affect penetration. The temperature at which the inoculated leaf was incubated was the factor determining penetration.

Appressoria of C. graminicola have continued to retain their ability to germinate after being air-dried and stored for 500 days. Mycelium produced new growth after being air-dried and stored for 400 days, while conidia were not able to germinate after being

air-dried for 24 hours. Thus, appressoria may function as organs of survival from one growing season to the next.

Detached appressoria, seeded immediately on PSA, were able to germinate, but were unable to do so if air-dried following detachment from the surface on which they were formed. The loss of viability of detached appressoria during air-drying may be the result of injury sustained during their removal or, it may be the result of excessive dessication caused by the exposure of the thin-walled pore which appears on the side of the appressorium that is in contact with the material to which it adheres. Detached appressoria when placed immediately on excised leaves penetrated directly or, in some instances, produced a germ tube which in turn produced another appressorium.

Thus, appressoria of this fungus are able to function as organs of penetration or as organs of survival analogous to the chlamydospores. They may be formed while the plant is resistant and remain as a latent source of infection until the plant is susceptible to penetration. Appressoria of several Colletotrichum spp. have been recognized as a source of latent infection (47, 49).

Appressoria of C. graminicola have the following chlamydospore-like characteristics: thick walls, resistance to long periods of dessication and the ability to produce germ tubes as well as penetration pegs. Although appressoria may function as chlamydospores, Dey (10) observed that the appressoria of C. gloeosporoides were not resistant to drying even when attached to the host. He concluded that, since appressoria of this fungus were unable to withstand drying, they could not be considered as organs of survival. Birachi (3), studying

the function of appressoria in Gloeosporium olivarum, concluded that they could function as chlamydospores. He proposed that these structures were analogous in function to chlamydospores, and contended that appressoria were produced as a result of a shortage of nutrients, including oxygen or as a defensive response to drying. Simmonds (47), working with Gloeosporium spp. that infect tropical fruit, found that appressoria were resistant to drying. He considered that their main function was to provide a firm attachment from which penetration might proceed, but that they could also be regarded as homologous to chlamydospores in function. Appressoria may act as chlamydospores under certain conditions when the host is resistant to penetration but the term, appressorium, should be used in reference to its function as an organ of penetration.

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